



UNIVERSITY of
BRADFORD

Library

The University of Bradford Institutional Repository

<http://bradscholars.brad.ac.uk>

This work is made available online in accordance with publisher policies. Please refer to the repository record for this item and our Policy Document available from the repository home page for further information.

To see the final version of this work please visit the publisher's website. Access to the published online version may require a subscription.

Link to publisher's version: <https://doi.org/10.1016/j.tox.2017.03.013>

Citation: Habas K, Brinkworth MH and Anderson D (2017) Diethylstilbestrol induces oxidative DNA damage, resulting in apoptosis of spermatogonial stem cells in vitro. *Toxicology*. 382: 117-121.

Copyright statement: © 2017 Elsevier. Reproduced in accordance with the publisher's self-archiving policy. This manuscript version is made available under the [CC-BY-NC-ND 4.0 license](https://creativecommons.org/licenses/by-nc-nd/4.0/).



Diethylstilbestrol induces oxidative DNA damage, resulting in apoptosis of spermatogonial stem cells *in vitro*

Division of Medical Sciences, Faculty of Life Sciences, University of Bradford,
Bradford, Richmond Road, West Yorkshire, BD7 1DP, UK.

Khaled Habas, Martin. H. Brinkworth, Diana Anderson

* To whom correspondence should be addressed:

Tel: + 44 (01274) 233569, E-mail: d.anderson1@bradford.ac.uk

Keywords: Diethylstilbestrol, DNA damage, quercetin, spermatogonial stem cells, apoptosis.

Highlights

- Exposure of the spermatogonial stem cells to DES produced significant increases in superoxide anion, DNA damage and apoptosis.
- The male reproductive system can be disrupted by foetal exposure to DES.
- The flavonoid quercetin reduced intracellular superoxide anions induced by DES.

Abstract

The spermatogonial stem cells (SSCs) are the only germline stem cells in adults that are responsible for the transmission of genetic information from mammals to the next generation. SSCs play a very important role in the maintenance of progression of spermatogenesis and help provide an understanding of the reproductive biology of future gametes and a strategy for diagnosis and treatment of infertility and male reproductive toxicity. Androgens/oestrogens are very important for the suitable maintenance of male germ cells. There is also evidence confirming the damaging effects of oestrogen-like compounds on male reproductive health. We investigated the effects in vitro, of diethylstilbestrol (DES) on mouse spermatogonial stem cells separated using Staptut unit-gravity velocity sedimentation, evaluating any DNA damage using the Comet assay and apoptotic cells in the TUNEL assay. Immunocytochemistry assays showed that the purity of isolated mouse spermatogonial cells was 90%, and the viability of these isolated cells was over 96%. Intracellular superoxide anion production (O_2^-) in SSCs was detected using p-Nitro Blue Tetrazolium (NBT) assay. The viability of cells after DES treatment was examined in the CCK8 (cell counting kit-8) cytotoxicity assay. The results showed that DES-induced DNA damage causes an increase in intracellular superoxide anions which are reduced by the flavonoid, quercetin. Investigating the molecular mechanisms and biology of SSCs provides a better understanding of spermatogonial stem cell regulation in the testis.

1. Introduction

Several compounds in the environment particularly those with estrogenic activity, are able to disrupt the programming of endocrine signalling pathways recognized through development; these compounds are referred to as endocrine-disrupting compounds (Schug *et al.*, 2011). Changed programming can result in numerous adverse consequences in estrogen-target tissues, some of which may not be apparent until later in life (Newbold, 2011). An *in vitro* study by Anderson *et al.* (Anderson *et al.*, 2003) showed that oestrogens produce reactive oxygen species (ROS), including hydrogen peroxide, at levels that disrupt DNA structure significantly in human sperm and lymphocytes. DES is a synthetic non-steroidal oestrogen that has been used as an extremely effective agent for androgen deprivation therapy in patients with prostate cancer (Kalach *et al.*, 2005; Koong and Watson, 2014), particularly in those who do not respond to other treatments, or those who are castrate-resistant (Grenader *et al.*, 2014; Youn *et al.*, 2012).

DES can bind to estrogen receptors (ERs) in the pituitary and has been widely used as a model estrogen to study alterations in male reproductive function in response to estrogens (Goyal *et al.*, 2001). It also has been shown that DES-induced many abnormalities in the male reproductive system are associated with changed DNA methyltransferase expression and DNA methylation (Sato *et al.*, 2006). DES can mimic estrogen action by interfering with the functioning of the pituitary-gonadal axis, leading to the suppression of testosterone levels that result in increased spermatogenic cell apoptosis (Akingbemi and Hardy, 2001). DES induced germ cell death is a suitable model to study the pathways involved in estrogen induced germ cell apoptosis (Nair and Shaha, 2003). An environmental study has confirmed that exposure to DES caused cancer and functional alterations in the reproductive

endocrine system in male and female mice treated *in utero* (McLachlan, 2016). It also showed that DES stimulation of cell proliferation and gene expression during the binding of the estrogen receptor has been implicated as the inducer of abnormalities caused by estrogens (McKinnell *et al.*, 2001). It has been found that acute exposure to DES induces apoptosis in the testis, and antioxidants play a role in preventing DES-induced testicular tissue damage (Kondo *et al.*, 2002). Studies from our laboratory have shown that quercetin acts as an antioxidant (Cemeli *et al.*, 2004; Dobrzynska *et al.*, 2004) and is an effective free radical scavenger; however, this is the first study where the cytoprotective effect of quercetin on DES-induced toxicity has been studied in isolated spermatogonial stem cells in mouse.

2. Materials and Methods

2.1. Animals

Male adult National Medical Research Institute (NMRI) mice (aged=6-8 weeks old; n=25), derived from the original stocks obtained from the Institute of Cancer Therapeutics Laboratories, were maintained under the standard conditions with free access to food and water at the Animal Facility of the University of Bradford, UK. All animal care procedures were carried out according to the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2. Isolation and culture of mouse spermatogonial cells

The method for isolation of spermatogonial cells was described previously for the mouse (Habas *et al.*, 2014). Briefly, six testes were collected from three adult NMRI mice (10-12 weeks old), decapsulated, and the seminiferous tubules placed into ice cold Dulbecco's Modified Eagle's medium (DMEM), dispersed by gentle pipetting,

minced and resuspended in fresh DMEM containing collagenase (5mg/ml) and DNase (1µg/ml) (both from Sigma, Poole, UK), then incubated at 32°C for 20 min. A second digestion step was performed in DMEM media by adding fresh enzyme solution into the seminiferous fragments. The cells were left to stand for 5 min before being filtered through an 80µm nylon mesh (Tetco Inc., Briarcliff Manor, NY), centrifuged at 600 × *g* for 10 min and bottom-loaded into the separation chamber of a Staput apparatus in a volume of 10 ml. A 2-4% w/v concentration gradient of Bovine serum albumin (BSA) was then generated below the cells, which were allowed to sediment for a standard period of 2.5 hrs. The cells in each fraction were examined under a phase contrast microscope, and consecutive fractions containing cells of similar size and morphology spun down by low-speed centrifugation and resuspended in DMEM.

2.3. Identification and morphological characteristics of mouse spermatogonial cell

The identity and purity of all cell preparations used in the experiments were confirmed by the immunohistochemistry assay for phase-specific markers exactly as described previously (Habas et al ., 2014). The purity of each population was also confirmed by western blot for the presence or absence of spermatogonia, spermatocyte and spermatid-specific proteins across the 3 independent experiments that were performed as described previously (Habas *et al.*, 2016).

2.4. Determination of cytotoxicity by cell counting kit-8 (CCK-8) assay

Cell viability was determined using a modified cell counting kit-8 (CCK-8) Cytotoxicity Assay (Sigma-Aldrich, UK). Spermatogonial cells were plated in a 96-well plate at a concentration of 5000 cells per well. 10 µl of different concentrations of DES (0, 1, 5 and 10 µM) was added into the culture media in the plate. Cells were pre-incubated

for 24 hrs in a humidified incubator at 37 °C, 5% CO₂. 10 µl of CCK-8 solution was added to each well of the plate, followed by incubation at 37 °C for 4 h. Absorbance was measured at a wavelength of 450 nm using a Microplate reader MRX II (Dynex Technologies, Chantilly, USA).

2.5. The Comet assay

The DNA damage was evaluated by the alkaline version of the Comet assay (pH>13) in spermatogonial cells after treatment with different concentrations of DES and quercetin. Approximately 2×10^5 cells per well were incubated with DES at final concentrations of 0, 1, 5 and 10 µM with or without 40 µM quercetin for 1 hr in eppendorf® tubes (5% CO₂, 95%, 37°C). Cell debris was removed by centrifugation and remaining cells were resuspended in 0.5% low melting agarose (LMP Agarose). The pellets from each treatment were harvested by centrifugation and then used for the evaluation of DNA damage using the Comet assay as described previously (Habas et al., 2016). Olive tail moment (OTM) and % tail DNA were measured, calculated, quantified and expressed (fold of control) in mean \pm SEM (n=3) for isolated spermatogonial cells using the (Comet 6.0; Andor Technology, formerly Kinetic Imaging) software image analysis system, Belfast, UK.

2.6. TUNEL staining and quantitation

The apoptotic cells were evaluated on isolated spermatogonial cells by the TUNEL assay using a commercial apoptosis detection kit (Terminal Deoxynucleotidyl Transferase Detection Kit; Promega, UK, Ltd). In brief, approximately 2×10^5 cells per well were incubated with DES at final concentrations of 0, 1, 5 and 10 µM with or without 40 µM quercetin at 37 °C for 1 h. The coverslips were incubated with TUNEL reaction mixture (30 mM Tris pH 7.4; 140 mM sodium cacodylate; 1 mM cobalt

chloride; 5 μ M biotin-16-deoxyuridine triphosphate; 0.3 U/ μ l terminal deoxynucleotidyl transferase [TdT]; all from Sigma) for 60 min (humidity chamber, 37 °C) and then washed twice in PBS. Cells were blocked with 0.03% H₂O₂. Cells were washed three times with PBS followed by treatment with 2% v/v Extravidin peroxidase in TBS with 0.1% w/v BSA for 30 min (humidity chamber, 37 °C). Cells were then washed again with PBS, and visualised by adding 3, 3-diaminobenzidine (DAB) for 10 min at room temperature. Cells were washed in phosphate buffered saline (PBS); each section counterstained with haematoxylin, then examined and photographed under an Olympus CKX31 microscope (Olympus, Southend on Sea, UK). Negative controls were obtained by incubating sections with the reaction mix in the absence of TdT. Marked condensation of chromatin and cytoplasm clearly staining strongly brown or brown/black were considered as apoptotic cells. The TUNEL positive cells were scored in around 10 fields on coverslips to yield a total of at about 100 cells under 400x magnification.

2.7. Detection of intracellular superoxide anion production

Spermatogonial cells were plated onto 24 well cell culture plates in complete DMEM medium at a density of 5×10^4 cells per well and incubated at 37°C and 5% CO₂. The cells were washed twice with PBS to remove any dead cells from the plate then spermatogonial cells were treated with 0, 1, 5 and 10 μ M DES for 1 hr. Medium then was aspirated and replaced with phenol red-free media (M199) (Sigma-Aldrich, UK), containing 1 mg/ml NBT with and without 1, 5 and 10 μ M DES. The cells then were incubated at 37°C and 5% CO₂ for 90 min. Spermatogonial cells were directly lysed with lysis solution (90% DMSO, 0.1% SDS and 0.01 M of NaOH). The resulting blue coloured solution was measured spectrophotometrically at a wavelength of 750nm using microplate reader MRX II (Dynex Technologies, Chantilly, USA). The

production of intracellular superoxide anion was proportional to the absorbance values at 750nm.

2.8. Statistical analysis

Data are expressed as mean \pm SEM of at least three independent experiments with three replicates per experimental group. Comparisons were made by one-way ANOVA followed by Bonferroni post-hoc test; for all experiments, a p value of <0.05 was considered significant.

3. Results

3.1. Cytotoxicity Assay

The cytotoxicity assay using CCK-8 kit was performed to directly determine the effect of DES on cellular viability of spermatogonia under our laboratory conditions. Spermatogonia were either treated with different concentrations of DES (0, 1, 5 and 10 μ M) or left untreated and considered as control. The result showed that DES at the concentrations 0, 1 and 5 μ M had no significant effect on cell viability. In contrast, at 10 μ M there was a significant effect on cell viability of spermatogonial cells ($*p \leq 0.05$) at 24hrs (Fig.1).

3.2. Detection of DNA damage by the Comet assay

Olive tail moment (OTM) and %tail DNA are shown in Figs. 2 and 3 respectively. A significant increase from 0.92 in control to 3.73 (OTM) and 7.23 in control to 21.29 (% tail DNA) was observed in the OTM and % tail DNA of spermatogonia treated with 5 μ M DES. At 10 μ M, OTM and % tail DNA damage also showed a significant increase to 6.34 and 26.10% respectively in spermatogonial cells compared with control ($**p \leq 0.01$ and $***p \leq 0.001$ respectively). Spermatogonial cell treated with 5

μM DES and 40 μM quercetin showed significant decreases in OTM and % tail DNA from 3.73 to 1.85 (OTM) and 21.29 to 11.53 (% tail DNA) respectively ($^{\#}p \leq 0.05$). At 10 μM , OTM and %tail DNA damage also showed a significant decreases from 6.34 to 2.09 and 26.10 to 13.00% respectively in spermatogonial cells compared with treated cells with 10 μM of DES alone ($^{###}p \leq 0.001$ and $^{##}p \leq 0.01$ respectively).

3.3. Detection of apoptosis by the TUNEL assay

The result of DES treatment on isolated spermatogonial cells of mouse testis was expressed as mean percentage per group \pm SEM of apoptotic cells. The TUNEL assay showed that spermatogonial cells had undergone significant levels of apoptosis compared with the controls ($^*p \leq 0.05$) (Fig. 4). After treatment with DES (0, 1, 5 and 10 μM) for 1h, a significant increase ($^*p \leq 0.05$) in spermatogonial apoptosis from 6.33 in control to 10.00 % was observed when cells were treated with 5 μM DES. This increased to 14.33 % when treated with 10 μM DES ($^*p \leq 0.05$). Spermatogonial cell treated with 5 μM DES and 40 μM quercetin showed that quercetin treatment resulted in a significant reduction in apoptotic cells from 10.00 to 6.67 compared with 5 μM DES alone ($^{\#}p \leq 0.05$). At 10 μM DES and 40 μM quercetin, a significant reductions from 14.33 to 9.00 was observed when compared with 10 μM DES alone ($^{##}p \leq 0.01$).

3.4. Detection of intracellular superoxide anion production

The NBT assay was used specifically to measure superoxide anion generation. Cells treated with DES for 1hr showed clear increased production of superoxide anions which were significantly increased compared to non-treated cells ($^*p < 0.05$), ($^{**}p < 0.01$) and ($^{***}p < 0.001$) (Fig. 5). Therefore, co-treatment with quercetin and DES was studied in spermatogonial cells in order to investigate whether generation might

reduce the increases in the generation of superoxide oxidase anion production. Cells treated with 1µM DES and 40µM quercetin showed that quercetin treatment resulted in a significant reduction in O_2^- levels compared with 1µM DES alone (^{##} $p \leq 0.01$). Cells treated with 5 µM DES and 40µM quercetin also showed a significant reduction in O_2^- levels compared with 5 µM DES alone ([#] $p \leq 0.05$). Further reduction in O_2^- levels was also shown when cells were treated with 10 µM DES alone (^{##} $p \leq 0.01$), all results shown in Fig. 5.

4. Discussion

The aim of the present study was to evaluate the DNA damage induced by DES and apoptotic cells in the TUNEL assay, as well as intracellular superoxide anions using the NBT assay in isolated spermatogonial cells. This study was also performed to investigate effects of quercetin on oxidative damage that was induced by DES in cultured spermatogonial cells of mouse testis. A number of previous studies have shown that the toxic effects of environmental oestrogens on male reproductive health are commonly performed *in vivo*. There are limited toxicity data *in vitro* (Anderson, Schmid, Baumgartner, Cemeli-Carratala, Brinkworth and Wood, 2003; Li *et al.*, 2010). It has been shown that estrogens induce apoptosis in male germ cells, but the pathways leading to apoptotic death from exposure to estrogens has not been fully understood (Nair and Shaha, 2003). A number of studies showed that cellular apoptosis in the testis in different model systems has used total testicular tissue and not isolated germ cells to separate pathways leading to cell death (Eid *et al.*, 2002; Yin *et al.*, 2002).

Using isolated male germ cells may provide a completely different understanding, as the total testicular tissue is a heterogeneous organ composed of multiple cell types

(Nair and Shaha, 2003). In this present study present, spermatogonial cells were isolated and cultured *in vitro*. Our results showed that DES produced DNA strand breaks in a concentration dependent manner in spermatogonial cells. This induction of DNA strand breaks is significant in that it shows how DES affects normal cells which could cause genetic damage that may be lead to cancer. A previous study has shown that DES undergoes redox cycling producing DES quinone and oxygen radicals; resulting in the production of adducts (Anderson, Schmid, Baumgartner, Cemeli-Carratala, Brinkworth and Wood, 2003; Thomas *et al.*, 2004). These results are supported by our present data which showed that exposure to DES had statistically significant increased DNA damage which could be the result of the production of superoxide by DES redox cycling. Studies have shown that exposure to DES might generate ROS (Anderson, Schmid, Baumgartner, Cemeli-Carratala, Brinkworth and Wood, 2003) and increased lipid peroxidation in rat and hamster models (Ma *et al.*, 2008).

The NBT results showed that high levels of O_2^- were formed in spermatogonial cells treated with 10 μ M DES. DNA damage can be attenuated by antioxidants. This study focused on quercetin as an antioxidant. Quercetin is an important flavonoid that has multiple beneficial biological, pharmacological, and medicinal properties including anti-inflammatory and cytoprotective effects (Gonzalez-Gallego *et al.*, 2010). It has been shown to reduce oxidative stress-dependent damage in both *in vitro* (Anderson, Schmid, Baumgartner, Cemeli-Carratala, Brinkworth and Wood, 2003; Liu *et al.*, 2010), and *in vivo* (Barcelos *et al.*, 2011). O_2^- levels were reduced when the spermatogonial cells were simultaneously exposed to DES. O_2^- was significantly reduced in the concomitant treatment of cells with 1, 5 and 10 μ M DES and 40 μ M quercetin ($^{##}p<0.01$).

These results are interesting for various important reasons. Our study using the *in vitro* model of spermatogonial stem cells could make it easier to understand the mechanisms underlying spermatogonia cell differentiation. Extrapolating to humans will be very useful, in which experimental approaches are limited. Furthermore isolated male germ cells could greatly improve and make the actual procedures of assisted reproductive technology more efficient and develop alternative infertility treatments. This technique is not new but it is rapid and does not need excessive animal experimentation *in vivo*, in contrast to traditional techniques that employ histological morphology and fertility studies. Furthermore, since this study only involves short term culture, it enables detection of the earliest signs of toxicity. This makes it useful for germline genotoxicity for drugs and other compounds of interest, and hence of value in safety evaluation procedures.

Conclusions

The results presented in this study showed that DES can adversely damage the spermatogonial stem cells by increasing DNA damage, apoptosis and superoxide anions. They also showed that quercetin had a protective effect on spermatogonial cells against oxidative damage and apoptosis caused by DES, which is an important flavonoid-reducing ROS and reproductive toxicity can be caused by this environmental oestrogen.

Acknowledgement

The Sponsorship of the Libyan Government of a PhD studentship to Khaled Habas is gratefully acknowledged. The Sponsor played no part in the conduct of the work or the writing of the manuscript.

Conflict of Interest Statement

The corresponding author confirmed that the authors have no conflicts of interest with regard to the funding of this research.

References

- Akingbemi, B. T., and Hardy, M. P. (2001). Oestrogenic and antiandrogenic chemicals in the environment: effects on male reproductive health. *Ann Med* **33**(6), 391-403, Doi 10.3109/07853890108995952.
- Anderson, D., Schmid, T. E., Baumgartner, A., Cemeli-Carratala, E., Brinkworth, M. H., and Wood, J. M. (2003). Oestrogenic compounds and oxidative stress (in human sperm and lymphocytes in the Comet assay). *Mutat Res-Rev Mutat* **544**(2-3), 173-178, 10.1016/j.mrrev.2003.06.016.
- Barcelos, G. R., Grotto, D., Serpeloni, J. M., Angeli, J. P., Rocha, B. A., de Oliveira Souza, V. C., Vicentini, J. T., Emanuelli, T., Bastos, J. K., Antunes, L. M., Knasmüller, S., and Barbosa, F., Jr. (2011). Protective properties of quercetin against DNA damage and oxidative stress induced by methylmercury in rats. *Archives of toxicology* **85**(9), 1151-7, 10.1007/s00204-011-0652-y.
- Cemeli, E., Schmid, T. E., and Anderson, D. (2004). Modulation by flavonoids of DNA damage induced by estrogen-like compounds. *Environmental and molecular mutagenesis* **44**(5), 420-6, 10.1002/em.20071.
- Dobrzynska, M. M., Baumgartner, A., and Anderson, D. (2004). Antioxidants modulate thyroid hormone- and noradrenaline-induced DNA damage in human sperm. *Mutagenesis* **19**(4), 325-30.
- Eid, N. A. S., Shibata, M. A., Ito, Y., Kusakabe, K., Hammad, H., and Otsuki, Y. (2002). Involvement of Fas system and active caspases in apoptotic signalling in testicular germ cells of ethanol-treated rats. *Int J Androl* **25**(3), 159-167, DOI 10.1046/j.1365-2605.2002.00341.x.
- Gonzalez-Gallego, J., Garcia-Mediavilla, M. V., Sanchez-Campos, S., and Tunon, M. J. (2010). Fruit polyphenols, immunity and inflammation. *The British journal of nutrition* **104 Suppl 3**, S15-27, 10.1017/S0007114510003910.
- Goyal, H. O., Braden, T. D., Mansour, M., Williams, C. S., Kamaleldin, A., and Srivastava, K. K. (2001). Diethylstilbestrol-treated adult rats with altered epididymal sperm numbers and sperm motility parameters, but without alterations in sperm production and sperm morphology. *Biology of reproduction* **64**(3), 927-934, DOI 10.1095/biolreprod64.3.927.
- Grenader, T., Plotkin, Y., Gips, M., Cherny, N., and Gabizon, A. (2014). Diethylstilbestrol for the treatment of patients with castration-resistant prostate cancer: retrospective analysis of a single institution experience. *Oncology reports* **31**(1), 428-34, 10.3892/or.2013.2852.
- Habas, K., Anderson, D., and Brinkworth, M. (2014). Development of an in vitro test system for assessment of male, reproductive toxicity. *Toxicology letters* **225**(1), 86-91, 10.1016/j.toxlet.2013.10.033.
- Habas, K., Anderson, D., and Brinkworth, M. (2016). Detection of phase specificity of in vivo germ cell mutagens in an in vitro germ cell system. *Toxicology* **353-354**, 1-10, 10.1016/j.tox.2016.04.001.
- Kalach, J. J., Joly-Pharaboz, M. O., Chantepie, J., Nicolas, B., Descotes, F., Mauduit, C., Benahmed, M., and Andre, J. (2005). Divergent biological effects of estradiol and diethylstilbestrol in the prostate cancer cell line MOP. *J Steroid Biochem* **96**(2), 119-129, 10.1016/j.jsbmb.2005.02.012.

- Kondo, T., Goto, S., Ihara, Y., Urata, Y., Ikeda, S., Hishikawa, Y., Izumi, S., Shin, M., and Koji, T. (2002). Diethylstilbestrol attenuates antioxidant activities in testis from male mice. *Free Radical Res* **36**(9), 957-966, 10.1080/107157621000006617.
- Koong, L. Y., and Watson, C. S. (2014). Direct Estradiol and Diethylstilbestrol Actions on Early-Versus Late-Stage Prostate Cancer Cells. *Prostate* **74**(16), 1589-1603, 10.1002/pros.22875.
- Li, G., Ma, A., Shi, W., and Zhong, X. (2010). Quercetin protects hamster spermatogenic cells from oxidative damage induced by diethylstilboestrol. *Andrologia* **42**(5), 285-90, 10.1111/j.1439-0272.2009.00990.x.
- Liu, S., Hou, W., Yao, P., Zhang, B., Sun, S., Nussler, A. K., and Liu, L. (2010). Quercetin protects against ethanol-induced oxidative damage in rat primary hepatocytes. *Toxicology in vitro : an international journal published in association with BIBRA* **24**(2), 516-22, 10.1016/j.tiv.2009.03.006.
- Ma, A., Yang, X. Z., Wang, Z. X., Shi, D. Z., and Chen, Y. X. (2008). Adult exposure to diethylstilbestrol induces spermatogenic cell apoptosis in vivo through increased oxidative stress in male hamster. *Reproductive toxicology* **25**(3), 367-373, 10.1016/j.reprotox.2007.12.007.
- McKinnell, C., Atanassova, N., Williams, K., Fisher, J. S., Walker, M., Turner, K. J., Saunders, T. K., and Sharpe, R. M. (2001). Suppression of androgen action and the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol. *J Androl* **22**(2), 323-38.
- McLachlan, J. A. (2016). Environmental signaling: from environmental estrogens to endocrine-disrupting chemicals and beyond. *Andrology* **4**(4), 684-94, 10.1111/andr.12206.
- Nair, R., and Shaha, C. (2003). Diethylstilbestrol induces rat spermatogenic cell apoptosis in vivo through increased expression of spermatogenic cell Fas/FasL system. *J Biol Chem* **278**(8), 6470-6481, 10.1074/jbc.M209319200.
- Newbold, R. R. (2011). Developmental exposure to endocrine-disrupting chemicals programs for reproductive tract alterations and obesity later in life. *Am J Clin Nutr* **94**(6), 1939s-1942s, 10.3945/ajcn.110.001057.
- Sato, K., Fukata, H., Kogo, Y., Ohgane, J., Shiota, K., and Mori, C. (2006). Neonatal exposure to diethylstilbestrol alters the expression of DNA methyltransferases and methylation of genomic DNA in the epididymis of mice. *Endocrine journal* **53**(3), 331-7.
- Schug, T. T., Janesick, A., Blumberg, B., and Heindel, J. J. (2011). Endocrine disrupting chemicals and disease susceptibility. *The Journal of steroid biochemistry and molecular biology* **127**(3-5), 204-15, 10.1016/j.jsbmb.2011.08.007.
- Thomas, R. D., Green, M. R., Wilson, C., and Sadrud-Din, S. (2004). Diallyl sulfide inhibits the oxidation and reduction reactions of stilbene estrogens catalyzed by microsomes, mitochondria and nuclei isolated from breast tissue of female ACI rats. *Carcinogenesis* **25**(5), 787-791, 10.1093/carcin/bgg161.
- Yin, Y. Z., Stahl, B. C., Dewolf, W. C., and Morgentaler, A. (2002). p53 and Fas are sequential mechanisms of testicular germ cell apoptosis. *J Androl* **23**(1), 64-70.
- Youn, J. Y., Nguyen, A., and Cai, H. (2012). Inhibition of XO or NOX attenuates diethylstilbestrol-induced endothelial nitric oxide deficiency without affecting its effects on LNCaP cell invasion and apoptosis. *Clin Sci* **123**(7-8), 509-518, 10.1042/Cs20110401.

Figure Legends

Fig. 1 Assessment of cytotoxicity of different concentrations of DES for 24hrs on spermatogonial cells using the cytotoxicity assay (CCK-8).

Fig. 2 Induced DNA damage in spermatogonial cells after treatment with different concentrations of DES (1, 5 and 10 μ M) alone or with 40 μ M quercetin for 1h. OTM was used for DNA damage quantification. * P <0.05, ** P <0.01, *** P <0.001 when compared with the respective control group. # p <0.05, and ### p <0.001 indicates cells treated with 1, 5 and 10 μ M DES and 40 μ M quercetin for 1hr.

Fig. 3 Induced DNA damage in spermatogonial cells after treatment with different concentrations of DES (1, 5 and 10 μ M) alone or with 40 μ M quercetin for 1h. %Tail DNA was used for DNA damage quantification. * P <0.05, ** P <0.01, *** P <0.001 when compared with the respective control group. # p <0.05, and ## p <0.01 indicates cells treated with 1, 5 and 10 μ M DES and 40 μ M quercetin for 1hr.

Fig. 4 Effect of DES treatment on spermatogonial cells assessed using the TUNEL assay. Data shows the mean percentages \pm SEM of apoptotic cells at DES concentrations of 1, 5 and 10 μ M alone or with 40 μ M quercetin for 1h. * P < 0.05, ** P < 0.01, *** P < 0.001 when compared with the respective control group. # p <0.05, and ## p <0.01 indicates cells treated with 1, 5 and 10 μ M DES and 40 μ M quercetin for 1hr.

Fig. 5 Effect of DES and quercetin co-incubation on superoxide anion production in spermatogonial cells using the NBT assay. Spermatogonial cells were grown in complete medium and treated with and without 1, 5 and 10 μ M DES alone or DES and 40 μ M quercetin for 1hr. Non-treated cells were considered as negative control. The results are expressed as mean (\pm SEM) from 3 different donors (n=3). * p <0.05,

**** $p < 0.01$ and *** $p < 0.01$ versus non-treated cells. # $p < 0.05$, and ## $p < 0.01$ indicates cells treated with 1, 5 and 10 μM DES and 40 μM quercetin for 1hr.**

Fig. 1

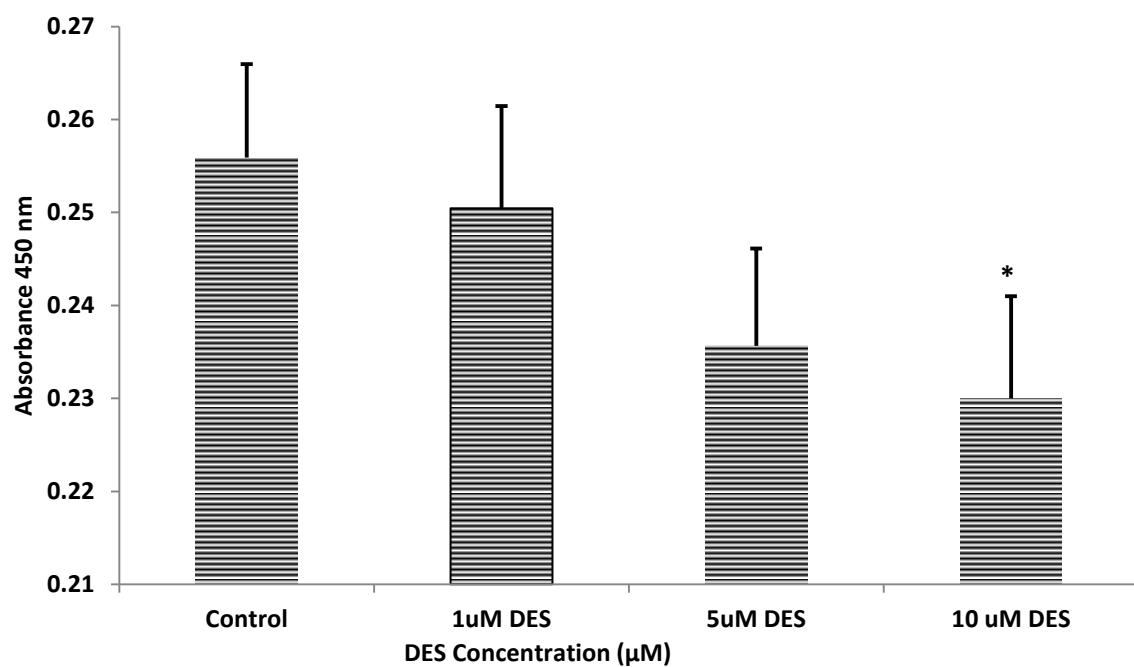


Fig. 2

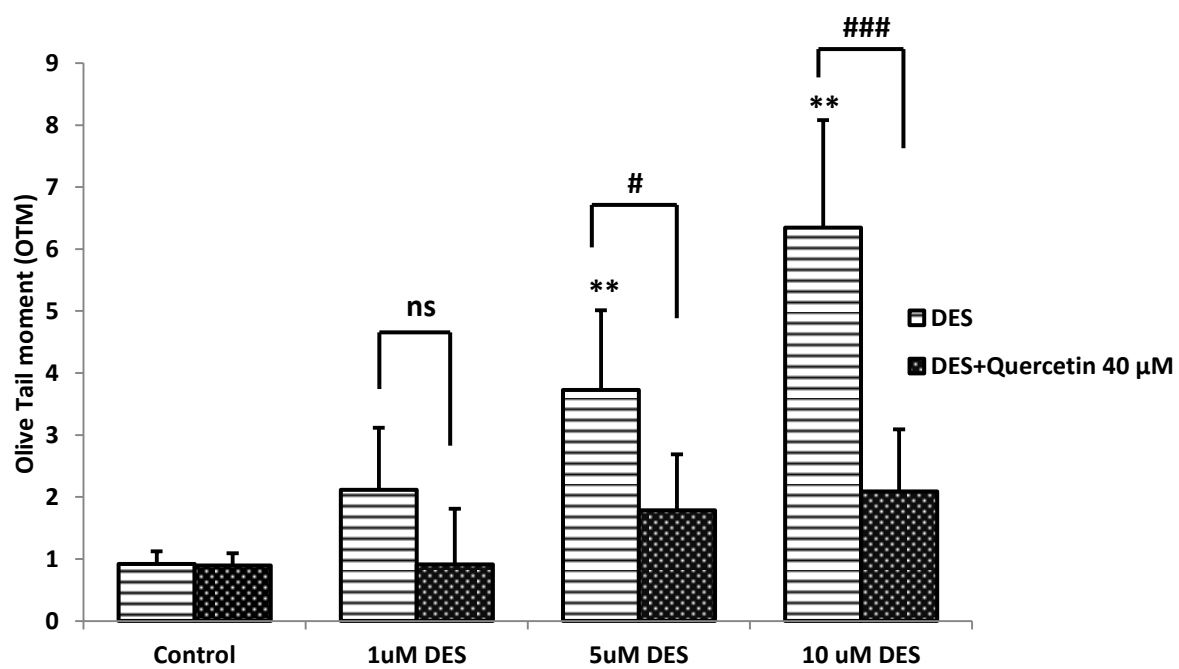


Fig. 3

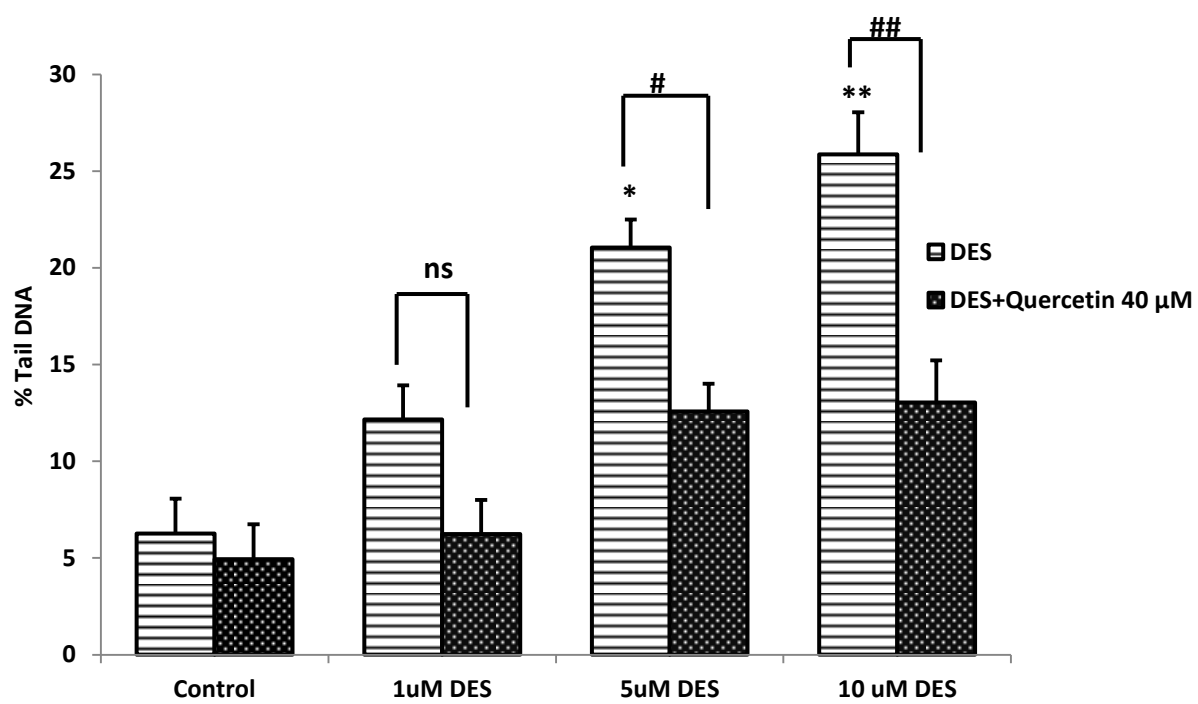


Fig. 4

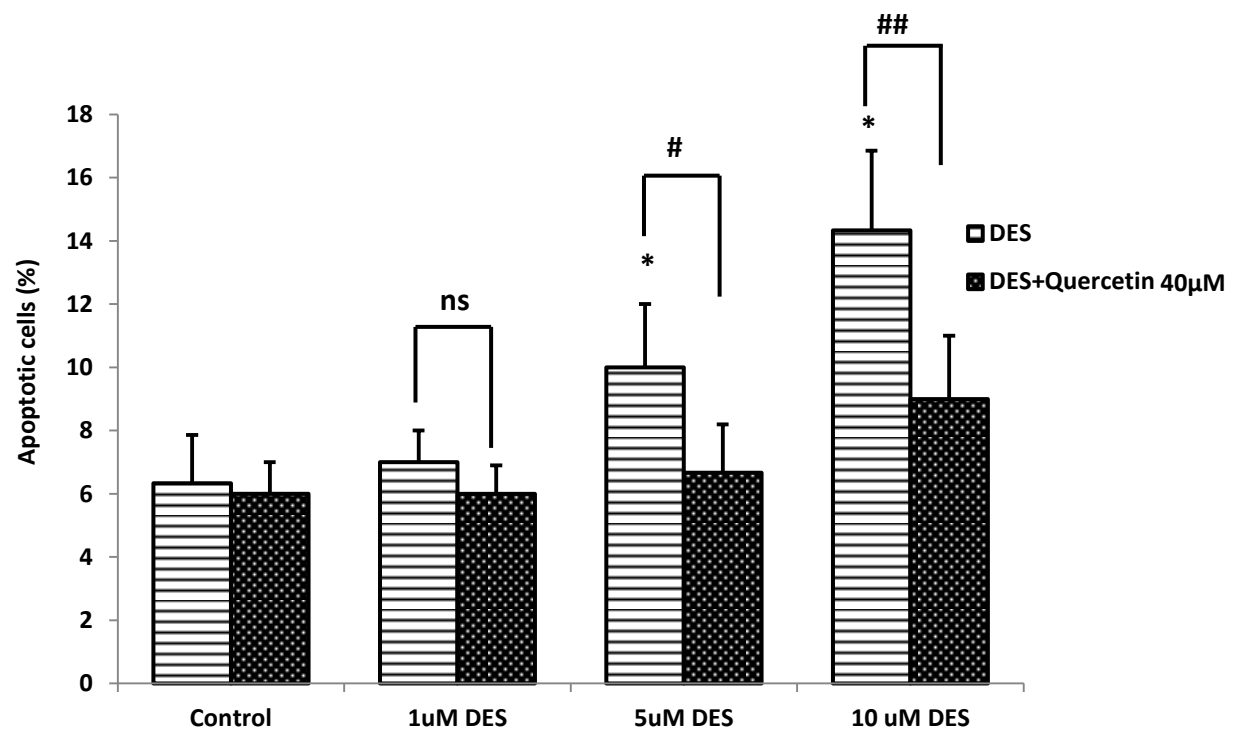


Fig. 5

